Canonical BMP signaling is dispensable for hematopoietic stem cell function in both adult and fetal liver hematopoiesis, but essential to preserve colon architecture


Division of Molecular Medicine and Gene Therapy, Lund Stem Cell Center, Lund University Hospital, Lund Sweden; || Present Address: St Vincent’s Institute of Medical Research, Fitzroy, Victoria 3065, Australia: *Harvard Stem Cell Institute, Cambridge, MA, USA; **Center for Genomic Regulation, ICREA and Pompeu Fabra University, Barcelona, Spain.

Corresponding author: Stefan Karlsson. Molecular Medicine and Gene Therapy, Lund University. BMC A12, 221 84 Lund, Sweden. Phone: +46 46 222 05 77, Fax: +46 46 222 05 78, E-mail: Stefan.Karlsson@med.lu.se

Running Title: BMP signaling is dispensable for HSC function
Abstract
Numerous publications have described the importance of Bone Morphogenetic Protein (BMP) signaling in the specification of hematopoietic tissue in developing embryos. Here we investigate the full role of canonical BMP signaling in both adult and fetal liver hematopoiesis using conditional knockout strategies, since conventional disruption of components of the BMP signaling pathway result in early death of the embryo. By targeting both Smad1 and Smad5, we have generated a double knockout mouse with complete disruption of canonical BMP signaling. Interestingly, concurrent deletion of Smad1 and Smad5 results in death due to extra-hematopoietic pathological changes in the colon. However, Smad1/5 deficient bone marrow (BM) cells can compete normally with wild-type cells and display unaffected self-renewal and differentiation capacity when transplanted into lethally irradiated recipients. Moreover, although BMP receptor expression is increased in fetal liver, fetal liver cells deficient in both Smad1 and Smad5 remain competent to long-term reconstitute lethally irradiated recipients in a multi-lineage manner. In conclusion, canonical BMP signaling is not required to maintain either adult or fetal liver hematopoiesis, despite its crucial role in the initial patterning of hematopoiesis in early embryonic development.
Introduction

During embryonic development, the hematopoietic system is one of the first complex tissues to form. Over the last two decades a significant number of studies have revealed the importance of Bone Morphogenetic Protein (BMP) signaling in mesoderm patterning, and the subsequent formation of hematopoietic cells in developing embryos (reviewed in\textsuperscript{1-4}). In this study we analyze the full role of canonical BMP signaling in both adult and fetal liver hematopoiesis. The necessity of BMP signaling in hematopoietic specification is largely conserved through evolution from Zebrafish and Xenopus\textsuperscript{5,6} to mouse and human\textsuperscript{7,8,9,10}. Targeted disruption of murine Bmp4, Bmp2, the type I receptor BmprIa or the type two receptor BmprII all result in early embryonic lethality and reduced formation of mesoderm\textsuperscript{7,8,11,12}. In particular BMP4 is important in regulating hematopoiesis, and has been reported to induce hematopoietic differentiation of murine and human ES cells \textit{in vitro}\textsuperscript{9,13}. Additionally, high concentrations of BMP4 maintains proliferation of human cord blood hematopoietic stem cells (HSCs) \textit{in vitro}\textsuperscript{10}, and also affects hematopoietic progenitors by inducing BFU-E formation of human CD34\textsuperscript{+} cells\textsuperscript{14}.

BMP is a member of the Transforming Growth Factor-\(\beta\) (TGF-\(\beta\)) superfamily. Signaling by these growth factors is initiated when binding of the ligand induces the assembly of a heteromeric complex of type I and type II serine/threonine kinase receptors. The type I receptors, also known as activin receptor-like kinases (ALKs), are recruited by the type II receptors and activate specific receptor-regulated Smads (R-Smads: Smad1-3, 5, and 8) by phosphorylation. Activated R-Smads then form complexes with the common-partner Smad4 and translocate to the nucleus where they participate in transcriptional regulation of target genes. Generally, Smad2 and 3 act downstream of the TGF-\(\beta\) and activin receptors, while Smad1, 5, and 8 primarily
mediate BMP signals.\textsuperscript{15,16}

In concordance with BMP ligand and receptor mutants, targeted homozygous disruption of the BMP signaling mediator \textit{Smad5} gene is also embryonic lethal. Smad5 deficient mice die between embryonic day 9.5 (E9.5) and E11.5, mainly due to defects in angiogenesis.\textsuperscript{17,18} Smad5 was shown to negatively regulate the proliferation and self-renewal of early multipotent hematopoietic progenitors derived from yolk sac and embryonic bodies \textit{in vitro}.\textsuperscript{19} Although there are some controversies regarding its role in differentiation,\textsuperscript{18,19} it can be concluded that Smad5 plays a role in murine embryonic hematopoiesis, while the role of the related Smad1 and Smad8 is largely unknown. In Zebrafish, however, loss of either \textit{Smad1} or \textit{Smad5} cause failure in generating definitive hematopoietic progenitors.\textsuperscript{20} Moreover, over-expression of Smad5 was unable to rescue the Smad1 loss-of-function phenotype. While the role of BMP signaling in hematopoiesis has mainly been examined in embryos, a recent study demonstrated an indirect effect of BMP on the number of adult murine HSCs. By conditionally knocking out BMP receptor Ia/ALK3 in adult mice Zhang et al. showed that impaired BMP signaling increases the niche size, and thereby enhances the number of HSCs.\textsuperscript{21} The same study demonstrated normal reconstitution ability of ALK3 deficient cells in \textit{wt} recipients, although this did not address the loss of BMP signals in HSCs intrinsically, as ALK3 is not expressed by these cells.\textsuperscript{21,22}

Since BMP signaling induces the hematopoietic stem cell program during embryonic development and also influences the HSCs via their niche, it may prove useful for generating and expanding the clinically important HSCs \textit{ex vivo}. As the Smads mediate signals from all of the different BMP ligands and receptors, and considering the involvement of Smad5 in hematopoietic regulation in the embryo, we have recently investigated the role of Smad5 in adult murine hematopoiesis using a
Cre-mediated conditional knockout model\textsuperscript{21} with the Cre-recombinase gene under control of the interferon inducible promoter \textit{Mx1 (MxCre)}.\textsuperscript{24} Surprisingly, HSCs lacking Smad5 display normal self-renewal and differentiation capacity following bone marrow (BM) transplantation, clearly indicating that Smad5 is dispensable for hematopoiesis in the adult mouse.\textsuperscript{25} Even though Smad1 and Smad5 possess inherent specificities, demonstrated by different expression and distribution patterns\textsuperscript{26-28} as well as dissimilar knockout phenotypes,\textsuperscript{17,18,27,29,30} it is conceivable that the absence of effects in our earlier study is due to redundancy with the related Smad1. Redundant mechanisms between Smad1 and Smad5 has recently been demonstrated as Smad1\textsuperscript{+/+}; Smad5\textsuperscript{+/+} murine mutants, in contrast to Smad1 or Smad5 single heterozygotes, are embryonic lethal and display defects that closely resemble those seen in Smad1 or Smad5 homozygous mutants.\textsuperscript{31} Additionally, Smad1 is able to rescue the Smad5\textsuperscript{-/-} phenotype in Zebrafish.\textsuperscript{20} Thus, Smad1 and Smad5 share equivalent functional activities in the early embryo.

To analyze the distinct versus overlapping roles of Smad1 and Smad5 in murine hematopoiesis, we created a conditional double knockout mouse for Smad1 and Smad5. Smad8 is not expressed in adult murine HSCs,\textsuperscript{22} and is therefore not likely to play a role in regulating HSC function. Surprisingly, our results show that both Smad1 and Smad5 are dispensable for normal hematopoiesis in adult mice, as Smad1/5 deficient BM cells can compete normally with wild-type (\textit{wt}) BM in transplantation settings and display unaffected self-renewal and differentiation capacity \textit{in vivo}. Additionally, fetal liver cells lacking both Smad1 and Smad5 are able to long-term reconstitute lethally irradiated recipients in a multi-lineage fashion, indicating that BMP signaling may be crucial only for the initiation of hematopoiesis during early embryonic development.
Materials and methods

Mice

Mice with “floxed” (fl) Smad1 or Smad5 alleles for Cre-mediated knockout have been previously described (loxP sites flank exon 2 in both Smad1 and Smad5).23,32 Using the described mice, we have generated a conditional Smad1/Smad5 double knockout on C57Bl6 background. Homozygously floxed single and double conditional knockout mice for Smad1 and Smad5 were crossed with heterozygote MxCre or VavCre mice to generate MxCre;Smad1fl/fl, MxCre;Smad1fl/fl/Smad5fl/fl, VavCre;Smad5fl/fl and VavCre;Smad1fl/fl/Smad5fl/fl offspring to study adult and fetal liver hematopoiesis respectively. Cre expression under influence of the Mx1-promoter was induced with 3 intra-peritoneal injections (at 2-day intervals) of 250 mg polyinositolic polycytidylic acid (pIC; Sigma-Aldrich St. Louis, MO). Cre under the influence of the Vav-promoter is constitutively expressed in hematopoietic cells from around E10.5.33 Genotyping for detection of wt, floxed, and excised alleles was done using a 3-primer polymerase chain reaction (PCR) as described previously.23,32 The presence of Cre was verified by PCR as previously described.25 Hetero- or homozygously “floxed mice” lacking Cre have two intact alleles of Smad1 and/or Smad5, and were hence used as wt controls. Mice were housed and bred in ventilated racks in a barrier facility. All animal experiments were approved by Lund University’s Animal Ethical Committee.

Histology

Induced MxCre;Smad1fl/fl/Smad5fl/fl mice and corresponding littermate wt controls were sacrificed when the double knockouts started to lose weight. Organs were fixed in PBS containing 4% paraformaldehyde, embedded, and subsequently sectioned and
stained with Erlish eosin for microscopic examination (Nikon Eclipse 50i, type: plan fluor, magnification: 100x, numerical aperture: 0.30).

**Cell preparations**

Peripheral blood (PB) was collected from the tail vein and analyzed on a blood cell analyzer (Sysmex, Boule Medonice CA 530-16) to determine cell counts. Bones and spleens were crushed and passaged through a 70-um cell strainer (Becton Dickinson (BD) Falcon, Bedford, MA) to obtain single-cell suspensions. Fetal livers were dissected out at E14.5, dissociated using a syringe, and filtered as above. Cells were kept in phosphate-buffered saline (PBS; Gibco-BRL, Paisley, UK) containing 2% fetal calf serum (FCS; Gibco-BRL). When necessary, red blood cells were lysed with ammonium chloride (NH₄Cl; Stem Cell Technologies, Vancouver, BC). For lineage depletion, cells were incubated with unconjugated rat antibodies against murine CD4, CD8, CD5, Gr1, Mac1, B220, and TER119 (BD Biosciences Pharmingen). Lineage-positive cells were removed with a magnetic particle concentrator (MPC-6; Dynal Biotech) following incubation with sheep anti-rat immunoglobulin G (IgG) crystallizable fragment (Fc)–conjugated immunomagnetic beads (Dynal Biotech, Oslo, Norway).

**Flow cytometry**

Rat antibodies against murine Mac1, B220, CD3, Sca-1, c-kit, CD34, FLT3, CD45.1, and CD45.2, either unconjugated, or conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or PECy7 were obtained from BD Biosciences Pharmingen. Unconjugated antibodies were detected with tricolor conjugated goat F(ab’)_2 anti-rat IgG (H+L) (Caltag Lab, Burlingame, CA). Dead cells
were excluded through staining with 7-aminoactinomycin D (7-AAD; Sigma-Aldrich, St Louis, MO). Cells were sorted by fluorescence-activated cell sorting (FACS) on a FACS Vantage Cell Sorter (BD) or analyzed on a FACS Calibur (BD). Results were analyzed with FlowJo software (Tree Star, Ashland, OR).

**Hematopoietic progenitor assays**

For granulocyte-macrophage colony-forming units (CFU-GMs) 30 000 BM or 15 000 fetal liver cells were plated per ml of methylcellulose medium (M3231; Stem Cell Technologies) containing 50 ng/ml murine Stem Cell Factor (mSCF; Amgen, Thousand Oaks, CA), 10 ng/ml murine Interleukin 3 (mIL-3; PeproTech, London UK), and 10 ng/ml human IL-6 (PeproTech). DNA from individual colonies was typed using PCR to check deletion efficiency.

**Liquid cultures**

Cells were grown in serum-free medium (StemSpan® SFEM (Stem Cell Technologies) or X-vivo 15; BioWhittaker, Walkersville, MD) supplemented with 1% BSA (Stem Cell Technologies), 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), 10^{-4} M 2-mercaptoethanol (Sigma-Aldrich), and 50 ng/ml mSCF (Amgen), 10 ng/ml mIL-3 and 10 ng/ml hIL-6 (Peprotech). Whole BM enriched for c-kit positive cells were grown at 5x10^5 cells/ml for 4 weeks. The cells were replated at 5x10^5 cells/ml and scored every third day. Lineage^−, Sca-1^+, c-kit^+ (LSK) CD34- cells enriched for HSCs were isolated by FACS (FACS Aria Cell Sorter; BD) and plated at one cell per well in 96-well plates containing 50 μl medium. 60 μl fresh supplemented medium containing cytokines was added at day 7 and proliferating cell clones were scored after 12 days (low proliferating<200 cells, high
proliferating>200 cells).

**Cell cycle analysis**

Freshly isolated BM cells were enriched for the c-kit+ population using magnetic beads (Fisher Scientific) and stained with antibodies for lineage markers, Sca-1, c-kit and CD34 prior to fixation using 0.4% formaldehyde (VWR) and permeabilized with 0.2% triton-X (Sigma). Permeabilized cells were subsequently incubated with anti-Ki67 antibody (BD Biosciences Pharmingen) and analyzed on a FACS Aria cell sorter (BD).

**Real time quantitative PCR (QRT-PCR)**

RNA isolation and cDNA synthesis were performed as previously described22 using sorted LSK CD34+ cells from the BM, or sorted Lin- Sca-1+ AA4.1+ cells from fetal liver. Quantification and normalization of RNA levels of different Smads and receptors involved in BMP signaling was also done as previously described,22 using the Taq-Man™ System and primers (Applied Biosystems, Foster City, CA).

**Transplantations**

In non-competitive transplantations of adult mice, 1x10^6 fresh BM cells from pIC induced MxCre;Smad1^fl/fl/Smad5^fl/fl or wt mice were transplanted, while 1x10^5 or 5x10^5 BM cells from induced MxCre;Smad1^fl/fl, MxCre;Smad1^fl/fl/Smad5^fl/fl and wt mice were transplanted together with 5x10^5 B6/SJL competitor BM cells in the competitive setting. When studying embryonic hematopoiesis 2x10^6 unfractionated E14.5 fetal liver cells from VavCre;Smad5^fl/fl, VavCre;Smad1^fl/fl/Smad5^fl/fl or wt embryos were transplanted without support cells. In all settings cells were
transplanted into lethally irradiated (900 cGy) recipient mice (2-3 recipients/donor). Donor, recipient, and support cells were tracked using congenic mouse strains expressing different isoforms of the pan-hematopoietic marker CD45 (CD45.1 versus CD45.2). Short-term hematopoiesis was analyzed 4-6 weeks post transplantation. After 16-40 weeks (long-term) recipients were sacrificed and half a femur–equivalent of BM was transplanted into lethally irradiated secondary recipients. PB was collected at several time points to determine donor reconstitution levels and lineage distribution by FACS analysis.

Statistical analysis
The significance of results was analyzed using the Mann-Whitney test (non-parametric), $p<0.05$ was considered significant.
Results

Concurrent deletion of Smad1 and Smad5, but not Smad1 alone, results in death due to pathology in the colon.

To study the effect of Smad1 and Smad1/5 deficiency during steady state hematopoiesis, MxCre;Smad1^{fl/fl}, MxCre;Smad1^{fl/fl}Smad5^{fl/fl} and wt mice were induced with pIC at 6-12 weeks of age, whereafter they were monitored for weight gain as an indicator of health. Smad1 deficient mice grew normally and remained healthy until the experiment was terminated at 16 weeks post induction (Figure 1A, data not shown), like we previously reported for Smad5 deficient mice.25 When analyzing hematopoietic parameters 8 weeks after induction, Smad1 deficient mice displayed unperturbed cell numbers and lineage distribution in PB, BM and spleen, except for a minor increase in B-cells in the spleen (Figure 1B). Smad1 KO mice also show normal numbers of LSK cells, a population enriched for hematopoietic stem and progenitors cells (Table 1).

When Smad1 and Smad5 were concurrently deleted (hereafter referred to as Smad1/5^{-/-}) the mice began to lose weight and most mice died within 4 weeks after induction (Figure 1A). All Smad1/5^{-/-} mice were anemic (Table 1) and tested positive for hemoglobin in the feces (data not shown), while this was not seen in any of the littermate controls. In addition to MxCre-induced gene deletion being highly efficient in the BM, the Mx1-promoter is also active in numerous other tissues, for example the gastro-intestinal tract.24 Several studies have previously shown that BMP signaling is crucial for maintenance of normal intestinal function.34,35 Additionally, when we conditionally knocked out the common Smad4 in a previous study, this resulted in a dramatic colon phenotype and intestinal hemorrhage, which was hypothesized to be due to disrupted BMP signaling.36 Accordingly, histological examination of the
gastrointestinal organs from induced \textit{wt} and \textit{MxCre;Smad1^{fl/fl}Smad5^{fl/fl}} mice revealed pathological changes in the colon submucosa of Smad1/5 deficient mice, including dilated crypts and occasional infiltration of inflammatory cells (Figure 1C). These mice also demonstrated a 4-fold increased spleen weight as compared to \textit{wt} controls (Figure 1D) with an accompanying increase in spleen cell numbers (Table 1), a phenotype also observed in the Smad4 deficient mice.\textsuperscript{36} The enlarged spleens displayed greatly expanded red pulp with prominent extramedullary hematopoiesis, consisting mainly of erythroid cells and megakaryocytes as determined by histological analysis (Figure 1E). The number of red blood cells was also substantially increased in the enlarged spleens (Table 1), suggesting that the displayed splenomegaly is a response to the intestinal bleeding. These results clearly confirm that the colon pathology seen in mice lacking Smad4 is due to disturbed BMP signaling. Moreover, FACS analysis of mature lineage committed cells in different hematopoietic organs from induced mice revealed that Smad1/5\textsuperscript{-/-} mice displayed a decrease in B-cells, and an increase in myeloid cells in the BM as compared to \textit{wt}, which was not detected in PB or spleen (Figure 1B).

\textit{Smad1/5 deficient BM cells display decreased colony forming ability in vitro}

To further study the features of hematopoietic stem and progenitor cells lacking Smad1 and Smad5, BM cells were isolated from \textit{wt} and \textit{VavCre;Smad1^{+/}Smad5^{+/}} littermates (the latter do not develop the lethal colon phenotype seen in induced \textit{MxCre;Smad1^{fl/fl}Smad5^{fl/fl}} mice). Both Smad1 and Smad5 were 100\% deleted in the HSC fraction (LSKCD34- cells) of these mice (data not shown). Importantly, the number of phenotypic long- and short-term HSCs, as well as lymphoid-primed multipotent progenitor cells remained unaltered in the absence of Smad1 and Smad5.
Additionally, freshly sorted long-term HSCs as well as multipotent progenitors from Smad1/5 knockout mice expressed similar levels of the proliferation marker Ki67 compared to littermate controls (Figure 2B). Furthermore, no significant difference in proliferation capacity was noticed between Smad1/5 deficient and wt cells, neither when sorted HSCs were seeded as single cells (Figure 2C), nor when the c-kit+ population, enriched for hematopoietic progenitors, from the same mice was cultured in bulk (Figure 2D). Together, this data demonstrate that Smad1/5 signaling is dispensable for HSC- and progenitor number, quiescence, as well as in vitro proliferation capacity. However, when plated in methylcellulose BM cells lacking Smad1 and Smad5 displayed a significantly decreased capacity to form myeloid colonies as compared to wt (Figure 2E).

**Smad1/5 deficient BM cells contribute to multi-lineage long-term reconstitution when transplanted into lethally irradiated recipients**

To restrict the Smad1 and Smad1/5 deletion to hematopoietic cells, BM from induced MxCre;Smad1fl/fl, MxCre;Smad1fl/flSmad5fl/fl and wt littermate mice was transplanted into lethally irradiated recipients. Importantly, hematopoietic cells deficient for both Smad1 and Smad5 demonstrated unperturbed proliferation and differentiation potential in vivo, and were able to reconstitute irradiated recipients in a multi-lineage fashion both short-term and long-term, as determined by FACS (Figure 3A-B). Recipients transplanted with Smad1/5−/− cells also displayed unperturbed numbers of total cells in the BM, as well as hematopoietic stem and progenitor cells per femur 5 months post transplant (Figure 3C). When examining the numbers of myeloid progenitors in vitro Smad1/5 deficient cells gave rise to similar numbers of colonies as wt cells, although the trend of decreased colony formation seen in the steady state
setting was still apparent in transplanted mice (Figure 3D). None of the mice transplanted with Smad1/5 deficient BM cells developed changes in the colon structure or spleen weight, indicating that the phenotype seen in the induced steady state mice is of extra-hematopoietic origin.

**Both Smad1 and Smad1/5 deficient HSCs compete normally with wt BM cells and display unaffected self-renewal and differentiation capacity**

To increase the pressure on the hematopoietic system, we mixed BM from wt, Smad1\(^{-/-}\) and Smad1/5\(^{-/-}\) mice with congenic wt BM at a 1:1 ratio, and transplanted the cell mixture into lethally irradiated recipients. As demonstrated in Figure 4, both Smad1 and Smad1/5 deficient BM cells were able to compete normally with wt cells, and contributed to long-term multi-lineage reconstitution of the recipients (Figure 4A-B). Furthermore, transplantation of BM from primary to secondary recipients revealed that HSCs from both Smad1 and Smad1/5 deficient mice could reconstitute the hematopoietic system after serial transplantation, and thus comprised normal self-renewal capacity, as well as differentiation ability (Figure 4C-D). Additionally, there was no significant difference in the number of primitive LSK cells in secondary recipients as assessed by FACS long-term after transplantation (data not shown). To avoid the risk of saturating the system by transplanting too many cells, we also performed a competitive transplantation where a reduced number of donor cells were competed against wt BM cells at a 1:5 ratio. However, in concordance with the findings from the other transplantations, the experiment revealed no difference between wt and Smad1/5\(^{-/-}\) hematopoietic repopulation (data not shown).

To confirm efficient deletion of the Smad1 and Smad5 gene respectively, individual hematopoietic colonies from wt, Smad1 and Smad1/5 deficient mice were
screened using PCR analysis (n>240 colonies, Figure 4E). Consistent with previous reports testing MxCre-mediated deletion of single genes in hematopoietic colonies, the targeted exon was deleted in 100% of the colonies tested from Smad1 single knockout mice. However, when concurrently knocking out Smad1 and Smad5, the deletion efficiency was on rare occasions slightly lower of one or both of the genes, although the absolute majority of the hematopoietic cells analyzed had a complete deletion in both genes. To control for that these rare hematopoietic cells with intact Smad1 or Smad5 expression did not take over and mask a potential phenotype, we sorted out donor derived hematopoietic BM cells 16 weeks after transplantation, plated them in methylcellulose and screened individual colonies for Smad1 and Smad5 deletion. Virtually all donor derived cells remained deficient in both Smad1 and Smad5, verifying that the few cells with remaining Smad1 or Smad5 did not out compete the Smad1/5 deficient cells.

BMP signals can be transduced by both Smad1, 5, and 8. Although Smad8 is not expressed in primitive hematopoietic cells in the BM we wanted to examine whether the expression levels of Smad8 was altered when knocking out Smad1 and Smad5, thus potentially generating a rescue mechanism. However, QRT-PCR analysis on sorted LSK cells from induced MxCre;Smad1<sub>1<sup>0/0</sup></sub>Smad5<sub>5<sup>0/0</sup></sub> and wt mice demonstrated that Smad8 remained undetectable also in primitive Smad1/5<sup>-/-</sup> BM cells (data not shown). Hence, the lack of phenotype in Smad1/5 deficient hematopoiesis in adult mice is not due to redundancy between related intracellular Smads.

**BMP signaling is dispensable for functional fetal liver hematopoiesis**

Numerous publications have described the importance of BMP signaling in the formation of hematopoietic tissue in developing embryos. Because targeted disruption
of the different components of the BMP signaling pathway all result in early death of the embryo.\textsuperscript{7,8,11,12,17} The majority of these studies are based on \textit{in vitro} experiments using very primitive hematopoietic cells derived from murine or human ES cells,\textsuperscript{9,13} murine yolk sac or embryonic bodies.\textsuperscript{18,19} We were therefore decided to study the role for BMP signaling in fetal liver hematopoiesis, a developmental phase in between the adult hematopoiesis in the BM and the primitive hematopoiesis found in yolk sac. To address the question of possible biological differences between these two ontogenic stages, the expression level of receptors involved in BMP signaling was analyzed by QRT-PCR in sorted primitive fetal liver cells. Intriguingly, the expression of ALK3 was markedly increased compared to levels in BM HSCs (Figure 5A), suggesting that there may be a discrepancy in response to BMP signaling between BM and fetal liver hematopoietic cells. As in the BM, Smad8 was not detectable in hematopoietic stem and progenitor cells derived from fetal liver.

Since deletion of Smad5 results in embryonic lethality at E10.5 due to defective angiogenesis, \textit{MxCre} cannot be used to study fetal liver hematopoiesis as this leads to deletion also in endothelial cells. Therefore, to study the deletion of the BMP mediating Smads in fetal liver cells we generated \textit{VavCre;Smad5\textsuperscript{fl/fl}} and \textit{VavCre;Smad1\textsuperscript{fl/fl}/Smad5\textsuperscript{fl/fl}} mice, where \textit{Vav} is a pan-hematopoietic gene. Driving \textit{Cre}-expression from the \textit{Vav}-promotor has been reported to cause deletion in essentially all hematopoietic cells including HSCs with an efficiency of 93-96\% in fetal liver, while leaving endothelial cells unaffected.\textsuperscript{33} Both \textit{VavCre;Smad5\textsuperscript{-/-}} and \textit{VavCre;Smad1\textsuperscript{-/-}/Smad5\textsuperscript{-/-}} mice were born at Mendelian ratios and were fertile. Fetal livers were dissected out on E14.5 and screened for deletion of respective Smad (Figure 5B). Surprisingly, when transplanted without support into lethally irradiated recipients, both Smad5 and Smad1/5 deficient fetal liver cells were capable of
reconstituting the hosts both short-term and long-term (Figure 5C). They were also able to generate all mature blood lineages at levels comparable to *wt* cells, apart from a slight but significant reduction of myeloid cells in PB from mice receiving Smad1/5−/− cells (Figure 5D). This decrease in myeloid cells was, however, not seen in the BM, either when analyzing lineage distribution by FACS (Figure 5E), or when examining the number of myeloid colony forming progenitors (Figure 5F). Additionally, mice receiving either Smad5−/− or Smad1/5−/− BM cells exhibited normal counts of red and white blood cells (data not shown), and displayed normal numbers of total BM cells as well as primitive LSK cells per femur long-term post transplantation (Figure 5G). BM cells from primary recipients were plated in methylcellulose 18 weeks after transplantation, and screening of individual colonies revealed a persistent 100% deletion of both Smad1 and Smad5. All hematopoietic parameters measured above remained normal after transplantation of Smad5−/− and Smad1/5−/− cells into secondary recipients, demonstrating that these cells possess unaffected self-renewal capacity (data not shown). We therefore conclude that BMP-signaling mediated by Smad1 and Smad5 is dispensable for functional fetal liver hematopoiesis.
Discussion

A significant body of evidence states an important role for BMP in initiating hematopoietic development in the early embryo (reviewed in\textsuperscript{1-4}). Because targeted disruption of components of the BMP signaling pathway result in embryonic lethality, the role of BMP signaling in adult hematopoiesis is largely unknown. Still, judging from its role in the embryo, expectations have been expressed that BMP signaling may prove useful for generating and expanding clinically important HSCs \textit{ex vivo}. In accordance with other components of BMP signaling, Smad5 affects hematopoiesis in early embryonic development \textit{in vitro}\textsuperscript{18,19}. However, using a conditional knockout strategy we recently demonstrated that Smad5 is dispensable for normal hematopoiesis in the adult mouse\textsuperscript{25}. To further address the issue of possible redundancy between related BMP mediating Smads, and also potential biological differences between ontogenic stages, we created a double conditional Smad1/Smad5 knockout mouse coupled to different Cre-expression patterns, thereby enabling investigation of the full role of canonical BMP signaling in both adult and fetal liver hematopoiesis.

Our results demonstrate that concurrent deletion of Smad1 and Smad5 results in death due to intestinal hemorrhage and pathological changes in the colon. This is in agreement with previous publications describing an important role for BMP in maintaining colon homeostasis\textsuperscript{34-36}. Moreover, Smad1/5\textsuperscript{-/-} mice have a compensatory enlargement of the spleen, and display a decrease in B-cells and increase in myeloid cells in the BM. The latter findings indicate that simultaneous deletion of Smad1 and Smad5 may affect the differentiation capacity of hematopoietic progenitors. However, the disturbed lineage distribution in the Smad1/5\textsuperscript{-/-} mice at steady state might also be explained by the finding that these mice are moribund and are in a bad general
condition. Accordingly, none of the mice transplanted with Smad1/5\(^{-/-}\) BM displayed any of the described symptoms, supporting that the observed phenotype was caused by the diseased Smad1/5 deficient environment. These results confirm our previous findings, where conditionally knocking out Smad4 resulted in a very similar colon phenotype, which was then hypothesized to be due to disrupted BMP signaling.\(^{36}\)

Interestingly, since knocking out only Smad1 or Smad5 did not result in disruption of the colon architecture, these related Smads seem to play a redundant role in maintaining colon homeostasis.

As opposed to a previous study where Smad5 was implied to negatively regulate the proliferation of multi-potent progenitors derived from yolk sac and embryonic bodies \textit{in vitro},\(^{19}\) Smad1/5 deficient HSCs derived from adult BM displayed normal numbers with retained proliferation status and expansion capacity \textit{in vitro}, as well as \textit{in vivo}. In accordance, transplanted BM cells lacking Smad1/5 displayed unaffected self-renewal and differentiation capacity \textit{in vivo} and competed normally with \textit{wt} BM in both primary and secondary recipients, suggesting that Smad-mediated BMP signaling is dispensable for HSC function in adult hematopoiesis.

Although the vast majority of the hematopoietic progenitors screened lacked both Smad1 and Smad5, it could be argued that the lack of phenotype is due to the very few remaining cells still expressing \textit{wt Smad1} or \textit{Smad5}. By plating sorted donor cells and screening individual colonies for deletion of the floxed \textit{Smad} genes, we could verify that cells with persistent Smad1 or Smad5 expression were close to absent in BM after long-term post transplantation. Furthermore, we could not detect any differences in any hematopoietic parameters analyzed between mice having 100% deletion of both \textit{Smad1} and \textit{Smad5} and mice with slightly lower deletion efficiencies.

In the light of previous findings, our study suggests that the developmental
context is an essential factor in determining how cells respond to growth factors. Several groups have showed the importance of functional BMP signaling for the formation of mesoderm and initiation of hematopoiesis in the developing embryo.\textsuperscript{7,8,11,12} Additionally, Bhatia et al. has demonstrated that high concentrations of BMP4 maintains proliferation of human cord blood HSCs \textit{in vitro}.\textsuperscript{10} Interestingly, the same study demonstrated significant expression of ALK3 and ALK6 in HSCs derived from human cord blood, while the expression level of these BMP type I receptors in BM derived HSCs was very low to undetectable.\textsuperscript{10} The absence of these BMP receptors has also been documented in murine BM.\textsuperscript{22} Intriguingly, we show here that ALK3 is expressed in primitive fetal liver cells although ALK6 is still undetectable, indicating that this cell population might respond differently to BMP compared to BM cells. Unexpectedly, fetal liver cells deficient in both Smad5 and Smad1/5 were still able to long-term reconstitute lethally irradiated recipients in a multi-lineage manner. Together this implies that that ALK6 may be an important type I receptor for BMP signaling in hematopoietic cells, and that murine HSCs of very early ontogeny might reflect a unique population with a distinct response to growth factors.

We could demonstrate that the related Smad8 is not expressed in BM cells lacking Smad1 and Smad5, suggesting that redundancy between related Smads is not accountable for the lack of phenotype \textit{in vivo} observed in adult hematopoiesis, in spite of perturbed BMP signaling. We can, however, not rule out that crosstalk with other pathways might influence the readout of our experiments. The diverging result seen when blocking the entire TGF-\textbeta family signaling by over-expressing the inhibitory Smad7\textsuperscript{38} or knocking out the common Smad4\textsuperscript{36} suggests that this might be an option. It has for example been shown that crosstalk between BMP and Wnt signaling is vital during early embryonic development in Xenopus.\textsuperscript{39} Additionally, BMP has been
reported to activate alternative pathways such as the mitogen-activated protein kinase cascade in other cell types.\textsuperscript{40} A possible role for non-canonical Smad-independent BMP signaling in hematopoietic cells might explain the differences between the phenotype obtained in our study, and studies where the entire TGF-\(\beta\) signaling pathway was inhibited or components of the BMP signaling pathway upstream of the Smads have been removed. To address this further investigations are required.

In conclusion, it is well known that several components of the BMP signaling cascade, including Smad5, are important regulators of hematopoiesis during early embryonic development. By investigating the effect of concurrent deletion of Smad1 and Smad5 in both fetal liver and adult hematopoiesis, we have hereby covered the role of canonical BMP signaling throughout ontogeny of murine hematopoiesis. Our results demonstrate that, in contrast to its crucial role during the patterning of hematopoiesis in the embryo, Smad-mediated BMP signaling is dispensable for normal HSC function in both adult and fetal liver hematopoiesis.

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**Authorship Contributions and Conflict of Interest Statements**

Sofie Singbrant has designed and performed research, as well as written the manuscript. Göran Karlsson has designed experiments, performed laboratory work, analyzed data and contributed to writing the manuscript. Mats Ehinger has performed the histopathological analysis, and Karin Olsson, Pekka Jaako and Ken-ichi Miharada has performed laboratory work. Matthias Stadtfeld, and Thomas Graf have characterized and contributed with mice vital for this study. Stefan Karlsson has directed research, designed experiments and contributed to writing the manuscript. SS and GK contributed equally to this study.

None of the authors declare any competing financial interests.

**Correspondence**

Dr. Stefan Karlsson, Molecular Medicine and Gene Therapy, Lund University Hospital, BMCA12, 221 84 Lund, Sweden, OR Dr. Sofie Singbrant, St Vincent’s Institute of Medical Research, 9 Princes Street, Fitzroy, Victoria 3065, Australia
References


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<th>WBC PB (10^9/L)</th>
<th>Cells/Femur (10^6)</th>
<th>LSK/Femur (10^3)</th>
<th>Cells/Spleen (10^6)</th>
<th>RBC Spleen** (10^9)</th>
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Table 1. Steady State Hematopoiesis in pIC induced mice. WT (n=3-7), Smad1 KO (n=3) and Smad1/5 KO (n=4). RBC; red blood cell count, WBC; white blood cell count, LSK; Lineage-Sca-1+c-kit+ cells, n.a; Not applicable. Data presented as average ± SD *p<0.03 (**Spleens suspended in 10 ml PBS)
Figure legends

Figure 1. Induced deletion of Smad1 and Smad5, but not Smad1 alone, results in death due to pathology in the colon.

(A) Weight change after induced deletion of Smad1 (S1-/--) and Smad1/Smad5 (S1/5-/-). (B) Lineage distribution in BM (left), PB (middle) and spleen (right) of induced mice (B-cells; B220, T-cells; CD3, and myeloid cells; Mac-1). Data presented as mean ± SD, *p<0.05, **p<0.001 (n=7/3/4 for WT, S1-/- and S1/5-/- respectively). Light grey bars = WT, dark grey bars = S1-/--, black bars = S1/5-/-.

(C) Pathological changes in the colon submucosa of S1/5-/- mice (right) compared to WT (left) at 100x magnification. (D) Spleen weight presented as mean ± SD (n=7) (left), representative spleens from WT and S1/5-/- mice (right). (E) Representative histological photos of WT (left) and enlarged S1/5-/- spleens (right) at 100x magnification. The arrows indicate the red pulp of the spleen.

Figure 2. Concurrent deletion of Smad1 and Smad5 alters the colony forming ability of myeloid progenitors in vitro.

(A) Quantity of phenotypic hematopoietic stem and progenitor cells in WT and S1/5-/- mice, as determined by differential counts and FACS (LT-HSC; LSKCD34-Flt3low, ST-HSC; LSKCD34+Flt3low, LMPP; LSKCD34+Flt3high) (B) Relative fraction of dividing cells as defined by expression of Ki67 in LT-HSCs (LSKCD34-) and MPPs (LSKCD34+). Representative experiment using BM from one S1/5-/- mouse and a littermate control (left), and pooled data from three independent experiments (right). (C) Single cell proliferation culture of primitive LSKCD34- cells, and (D) bulk culture of c-kit enriched BM cells. (E) Quantity of myeloid progenitors, as determined by the number of colony forming cells (CFU-GM). Data presented as mean ± SD, *p<0.05 (n= 2-5). Light grey bars = WT, black bars = S1/5-/-.
**Figure 3. Smad1/5 deficient BM cells contribute to multi-lineage long-term reconstitution when transplanted into lethally irradiated recipients.**

(A) Contribution of donor cells in PB short-term (ST=4w) and BM long-term (LT=21w) after transplantation. (B) Distribution of myeloid cells (Mac-1), B-cells (B220) and T-cells (CD3) within the donor population in PB 21 weeks post transplantation presented as mean ± SD. (C) Number of total cells/femur, primitive LSK cells/femur and (D) myeloid progenitors in BM 21 weeks post transplantation, as determined by differential counts, FACS and the number of formed colonies (CFU-GM) respectively. (n=3 donors and 9 recipients). Light grey bars = WT, black bars = S1/5-/-.

**Figure 4. Both Smad1 and Smad1/5 deficient BM cells display normal self-renewal and differentiation capacity following competitive BM transplantation**

(A) Short-term contribution (ST) in PB and long-term (LT) reconstitution in BM in primary and (C) secondary recipients following competitive transplantation, as measured by Ly5.2/Ly5.1 contribution. (B) Distribution of myeloid cells (Mac-1), B-cells (B220) and T-cells (CD3) within the donor population in PB from primary and (D) secondary recipients analyzed by FACS long-term post transplantation. Data represents mean ± SD (n=9/5/8 donors and 25/15/19 recipients for WT, S1-/- and S1/5-/- respectively for primary transplantations, and n=7/5/3 donors and 21/15/9 recipients for WT, S1-/- and S1/5-/- respectively for secondary transplantations). Light grey bars = WT, dark grey bars = S1-/-, black bars = S1/5-/-.

(E) Representative PCR screen of individual hematopoietic colonies from WT and S1/5 deficient mice to determine deletion (total number of colonies screened n>240). Lane 1-4; floxed (no band) and deleted (300 bp) Smad1 in WT (1-2) and S1/5-/- colonies (3-4). Lane 5-8; floxed (2,3 kb) and deleted (387 bp) Smad5 in WT (5-6) and S1/5-/- colonies (7-8).
Figure 5. Both Smad1 and Smad5 are dispensable for functional fetal liver hematopoiesis

(A) Expression of BMP-receptors in WT fetal liver cells analyzed by QRT-PCR. ALK3 is expressed in Lin- Sca-1+ AA4.1+ fetal liver, but is undetectable in LSK CD34- BM cells.10,22 (B) Representative gel showing screening of Smad5 deletion (PCR) in fetal livers. Only livers with highly deleted Smad1 (data not shown) and Smad5 were used (such as #10). WT littermates lacking Cre have floxed, but undeleted genes (such as #1). (C) Short-term (ST) donor reconstitution in PB and long-term (LT) contribution in BM. Distribution of myeloid cells (Mac-1), B-cells (B220) and T-cells (CD3) within the donor population in PB (D) and BM (E) analyzed by FACS long-term post transplantation. Number of myeloid progenitors (F), total cells/femur and primitive LSK cells/femur (G) in BM long-term post transplantation as determined by the number of formed colonies (CFU-GM), differential counts, and FACS respectively. Data represents mean ± SD (n=5 donors and 15 recipients per genotype). Light grey bars = WT, dark grey bars = S5-/-, black bars = S1/5-/-.
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Canonical BMP signaling is dispensable for hematopoietic stem cell function in both adult and fetal liver hematopoiesis, but essential to preserve colon architecture

Sofie Singbrant, Göran Karlsson, Mats Ehinger, Karin Olsson, Pekka Jaako, Ken-ichi Miharada, Matthias Stadtfeld, Thomas Graf and Stefan Karlsson